DOI:10.1021/jf900127u

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Morphology, Associated Protein Analysis, and Identification of 58-kDa Starch Synthase in Mungbean (*Vigna radiata* L. cv. KPS1) Starch Granule Preparations

YUAN-TIH KO,* YU-LING DONG, YING -FANG HSIEH, AND JA-CHI KUO

Department of Food Science, Biotechnology Division, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung, Taiwan 20224

Raw starch granules of mature mungbean (*Vigna radiata* L. cv. KPS1) seeds were prepared by two methods into crude and cesium chloride (CsCl)-washed forms. The purity, shape, size distribution, and associated protein profiles were examined. The appearance of raw starch granules showed a bimodal type distribution in which average granules had typical ovoid shapes, whereas the small ones were spherical. Abnormal granule surface with distinct tumor-like or dented hole features were also observed in raw starch granules. CsCl-washed granules had a smooth surface compared to that of the crude form. The granule size distribution ranged from 6−35 μm; most 15−25 μm (~53%), followed by 25−35 μm (~26%). Small granules (<15 μm) amounted to ~18%, and granules >35 μm consisted of ~3%. The two forms were further refined by trichloroacetic (TCA) treatment to reveal surface proteins on the crude granules or tightly bound proteins on CsCl-washed granules. In the washed-refined granules, only a few integral proteins were retained. The major 58-kDa protein was identified to be granule-bound starch synthase I by sequence homology with that in cowpea (*Vigna unguiculata*) and maize (*Zea mays*) using MALDI-TOF mass and Mascot search.

KEYWORDS: *Vigna radiata*; starch granule; granule morphology; granule-associated protein; granule bound starch synthase

INTRODUCTION

Mungbean (*Vigna radiata* L.) is one of the common crops in the subtropical and tropical areas. Mungbean starch is an essential ingredient used in making starch noodles (or bean threads) and paste cakes. The total starch content of mature mungbean seeds, ranging from 49 to 60% of dry weight, correlated with the glossiness of the bean surface. Mungbeans were classified into shiny- or dull-seed types based on 159 mungbean cultivars studied, which showed that the glossier the surface appearance, the higher the starch content, the lower the protein content, and the higher the resistance to fungal infection (*1*). There were unique chemical features in mungbean starch such as high amylose level (up to 45% compared with normal 15−30%) and long amylose chain length inside amylopectin structure. These features determine the physicochemical properties of mungbean noodle to exhibit translucent appearance and resistance to shearing upon heating (*2*). The unusual resistance of mungbean starch to shearing and heat is also due to the presence of a small number of protein/peptide-cross-linked segments of the amylopectin (*3*).

The size and shape of starch granules are diverse among different plant species and genetic backgrounds. Granule size ranged from 1 to 100 μm in diameter. Multiple shapes are found in starch from natural sources including polygonal, round/spherical, lenticular, elliptical, truncated, ovoid, and compound. One source may have different shapes. The reasons for such differences in granules architecture in nature are not well understood, but indeed they affect the functional properties of starch granules (*4*, *5*). Mungbean starch granules are mainly of spherical and oval particles (*6*), which thus provide a unique smooth mouth-feel when its starch powder is made into bean paste cake. This attribute makes mungbean starch irreplaceable by other starch raw materials in much of the food manufacturing industry.

In addition to their starch content, starch granules contained an average of 0.25% protein, a group of granule-associated proteins (SGAPs) of tightly bound, surface, or integral proteins, and are biologically distinct from plant storage proteins in seeds or tubers. It is important to understand the identities, compositions, locations, and properties of SGAPs and their roles in the biogenesis of the granules structure (*7*). SGAPs were also shown to influence the rheological properties of corn starch pastes (*8*). To analyze the SGAPs of mungbean starch and further reveal their functions, it is important to prepare intact granules without contamination from storage proteins such as legumins (*9*). It is also important to avoid the loss of granule populations during preparation. Starch extraction, isolation, and the purity of a starch preparation are therefore, the key quality factors to investigate SGAPs.

Multiple methods have been reported for mungbean starch isolation and recovery of proteins from wastewater in the food industry (*6*, *10*). These methods were designed on the scale of manufacturing plants and intended to remove lipid/proteins to prepare clean uncontaminated mungbean starch granules and

*Corresponding author. Tel: (02) 2462-2192, ext. 5132. Fax: (02) 2463-4203. E-mail: irisko@ntou.edu.tw.

 Published on Web 4/16/2009

amylose/amylopectin fractions (*3*). However, they would not be suitable for laboratory-scale starch granule preparation, and limited information was available on the starch-associated proteins. Previously, we have detected starch synthase activities of the raw starch granules and in the amylase-digested lysate of mungbean KPS1 var. and analyzed protein profiles on the granule's surface (*11*). Accordingly, it is important to further understand mungbean starch granules and to analyze SGAPs. The specific objectives of this study are to examine the morphology of different granule preparations, and to find the associated proteins related to starch biosynthesis.

For this purpose, we prepared three forms of the KPS1 starch granules. The crude granule preparation was adopted from a previous method (*11*, *12*). The washed granule preparation was from a single kernel preparation method using cesium chloride (CsCl) wash (*13*), and the refined granule preparation was obtained by trichloroacetic (TCA) treatment on the crude and CsCl-washed granules. The granule morphology, size distribution, surface purity, and characteristics were examined by scanning electron microscopy (SEM), and SGAPs were analyzed by SDS−PAGE. A major integral protein in the washed−refined granules was identified by MALDI-TOF mass spectrometry and Mascot search.

MATERIALS AND METHODS

Mungbean KPS1 (*Vigna radiata* L., Kamphaeng Saen 1), a shiny type variety, was obtained from Asian Vegetable Research and Development Center (Shanhua, Tainan, Taiwan) and kept dry in a desiccator before use. This variety was distributed worldwide with the names of KPS1 in Taiwan and Thailand, Zhong Lu 1 in mainland China, Seonhwa-nogdu in Korea, Silangan in Philippines, and Tex-Sprout in the U.S. and Canada (*14*). Acetone, bovine serum albumin (BSA), bromophenol blue, CsCl, dimethyl sulfoxide (DMSO), glycerol, iodoacetic acid (IAA), 2-mercaptoethanol, potassium iodide (KI), sodium bisulfite ($Na_2S_2O_5$), TCA, and Tris-HCl were from Sigma Chemical Co. (St. Louis, MO, USA). Dithiotreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) were from USB (Cleveland, Ohio, USA). Iodine (I_2) was from Showa (Japan). Hydrochloric acid (HCl) was from Merck (Darmstadt, Germany). Ethanol was from Union Chemical Works Ltd. (Hsinchu, Taiwan). Chemicals for electrophoresis such as acrylamide, *N*′,*N*′-methylene bis-acrylamide, ammonium persulfate (APS), coomassie brilliant blue (Bio-Safe), sodium dodecyl sulfonate (SDS), and tetramethylethylenediamine (TEMED) were from Bio-Rad (Hercules, CA, USA). Broad range 10−200 kDa molecular marker was from Fermentas (PageRuler Unstained Protein Ladder, Vilnius, Lithuania).

Crude Granule Preparation. One gram of KPS1 mungbean (approximately 8 grains) was soaked in 10 mL of dH_2O at 4 °C overnight to remove the seed coat. The cotyledons were collected and ground in ice-cold 0.1% $Na_2S_2O_5$ solution (1/10 w/v) followed by filtering through 4 layers of cheese cloth with intermediate washing three times to obtain 30 mL of solution with a final DTT concentration of 1 mM. The solution was kept at room temperature for 1 h followed by centrifugation at 12000*g* at 4 °C for 30 min. The precipitate was washed successively with 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT once, 50 mM Tris-HCl (pH 7.5) three times, and −20 °C cold acetone three times. The resulting raw starch granules were air-dried and stored at −20 °C until analysis (*12*).

CsCl-Washed Granule Preparation. One grain of KPS1 mungbean was soaked in 10 mL of dH_2O at 4 °C for 1 day to remove the seed coat. The cotyledons were collected and ground in 1 mL of dH_2O at 4 °C and centrifuged 12000*g* at 4 °C for 30 min. The precipitate was extracted with 0.8 mL of 80% (v/v) CsCl and centrifuged 12000*g* at 4 °C for 30 min. The precipitate was washed successively with dH_2O three times, 1% SDS once, dH_2O three times, followed by filtration through a 60 μm Nylon filter (Millipore, Bedford, MA) by a water pump. The filtrate was centrifuged 14000*g* at 4 °C for 20 min, and the pellet was washed twice by −20 °C cold acetone. The resulting raw starch granules were air-dried and stored at −20 °C until analysis (*13*).

TCA-Refined Granule Preparation. TCA-refined starch granules were prepared by further TCA treatment on crude or washed granule forms. Three milligrams of starch granules were incubated successively with 1 mL of 130 mM DTT in TCA and 1 mL of 135 mM IAA in TCA solution at 4 °C for 10 min twice. During the procedures, granules were recovered by centrifugation at 14000*g* at 4 °C for 5 min. Granules were finally washed with 200 μL of ice-cold acetone, air-dried, and stored under −80 °C.

Gelatinized Starch Preparation. Dried crude raw starch of 0.5 g/mL in 90% DMSO (v/v) was gelatinized in a sealed tube by heating in a dry bath at 100 °C for 3 h. The solution was centrifuged 12000*g* at room temperature. The pellet was resuspended in 0.5 mL 80% (v/v) ethanol, centrifuged at 12000*g* for 30 min to decant solution, and centrifuged another 5 min to recover the pellet. The pellet was washed twice with −20 °C cold acetone and dried at 50 °C overnight, and stored at −20 °C until analysis.

Granule Morphology. Raw starch forms or gelatinized starch of 50 mg was added to 1 mL of Lugol's solution (10 mM I_2/14 mM KI) in a microcentrifuge tube, standing for 5 min. A drop of starch solution was withdrawn onto a slide, coverslipped, and photographed under a light microscope. For SEM, starch granules were stuck onto a stub and coated with a thin layer of gold for 1 min with a 1 min cooling interval 6 times. The prepared samples were examined with a Hitachi, S-2400 scanning electron microscope (Hitachi, Japan) at National Taiwan Ocean University Electron-Microscopy Center.

Granule-Associated Protein Analysis and Protein Identification. The dried starch granules in 5 mg/0.1 mL of extraction buffer (125 mM Tris-HCl at pH 6.8, 2.5% glycerol, 1.25% 2-mercaptoethanol, 0.5% SDS, and 0.05% bromophenol blue) were heated at 100 °C for 2 min followed by centrifugation at room temperature under 10000*g* for 2 min. The supernatant was subjected to discontinuous SDS−PAGE in a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA, USA). Separation gel was 10%, pH 8.8, and stacking gel was 4%, pH 6.8 polyacrylamide gel (acrylamide: bis, 37.5:1). Proteins were visualized by Silver-staining or Bio-Safe coomassie blue-staining (Bio-Rad). SGAP was excised from the coomassie blue stained SDS−PAGE gel and subjected to in-gel trypsin digestion and MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) analysis (*15*). The MALDI-TOF mass analysis was conducted by the Genomic Medicine Research Core Laboratory of Chang-Gung Memorial Hospital (Linkou, Taoyuan, Taiwan).

RESULTS

Morphology and Size Distribution of Mungbean Starch Granules under SEM. The granule surfaces in the crude granule preparation were covered with much more cellular debris (**Figure 1A**)

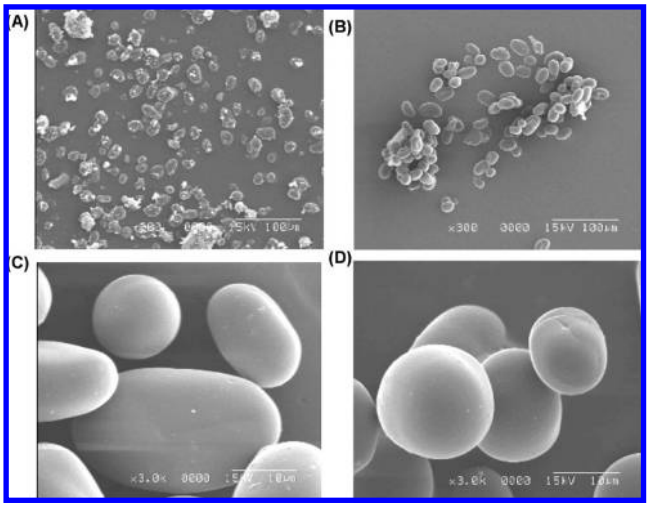

Figure 1. Scanning electron micrographs of raw starch granule preparations. (**A**) The raw granules; (**B**, **C**, **D**) the CsCl-washed starch granules. The operating voltage was 15 kV.

compared with that of the washed granules that had a clean and smooth appearance (**Figure 1B**). Mungbean granules exhibited a mixture of ovoid and spherical populations (**Figure 1C**,**D**). Size distribution of the crude granules ranged from 6 to 35 μm, while the washed granules ranged from 10 to 30 μm in diameter/length. The narrow size range of washed granules was due to filtration through a 60 μm nylon membrane during preparation. Most granules were in the 15−25 μm range (50% in the crude and 56% in the washed granules; average ~53%), followed by the 25−35 μm size range (29% in the crude and 23% in the washed granules; average ~26%). Small granules of less than 15 μm amounted to as much as 18% (crude granules) and 19% (washed granules).

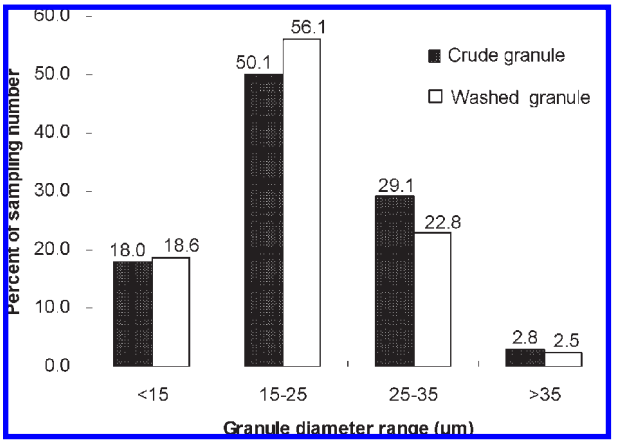

Figure 2. Size distribution of the raw and washed starch granule preparations. Sampling number was 500 granules for each. The raw granule preparation had been filtered by passing through four layers of cheesecloth, while the washed granule preparation had been filtered by passing through a Nylon filter followed by washing with 1% SDS detergent as described in Material and Methods.

The majority of small granules with sizes smaller than 15 μm were spherical in shape. Large granules with sizes bigger than 35 μm amounted to 2.8% (crude granules) and 2.5% (washed granules) (**Figure 2**). When the washed granules of different sizes were individually observed (**Figure 3**, pointed by arrows), it clearly showed that most granules had typical ovoid shapes, whereas the smallest 1/5 of total starch granules (< 15 μm) were spherical. It is thus a bimodal type of distribution, and the observation is consistent with a recent report on a commercial mungbean variety (*16*).

Special Features of Mungbean Starch Granule Morphology. SEM observation revealed an unusual appearance on a few mungbean granules. Ovoid-shaped granules were found to have tumor-like surfaces (**Figure 4A**) or dented holes (**Figure 3**, the 33 μm focused granule; **Figure 4B**). Some large ovoid-shaped granules were observed to have a fissured slot (**Figure 4C**) or growth-line texture (**Figure 4**D).

Comparison of the Raw and Gelatinized Starch Granules under a Light Microscope. Under a light microscope, raw starch granules also showed large ovoid and small spherical granules (**Figure 5A**). Iodine stained the granules dark blue, and the bimodal population type was evident (**Figure 5B**). When gelatinized granules were stained light blue, the shape deformed into irregular clumps (**Figure 5C**). Iodine molecules are known to form a complex with starch, possessing higher affinity with amylose than with amylopectin (*17*). The crystal structure of starch granules was dissolved into open α-glucan molecules of the gelatinized starch. As a result of the less-ordered fine amylose structure, and the exposure of the lipid-complex, which was originally located within the amylose, iodine was inhibited from binding with the amylase/amylopectin molecules, and the blue tone decreased.

SGAP Analysis in Granule Preparations. The surface of the washed and refined granules was smooth compared with that of the crude granule preparation. Using SDS−PAGE analysis of the hot SDS buffer-extracted fractions from the three granule

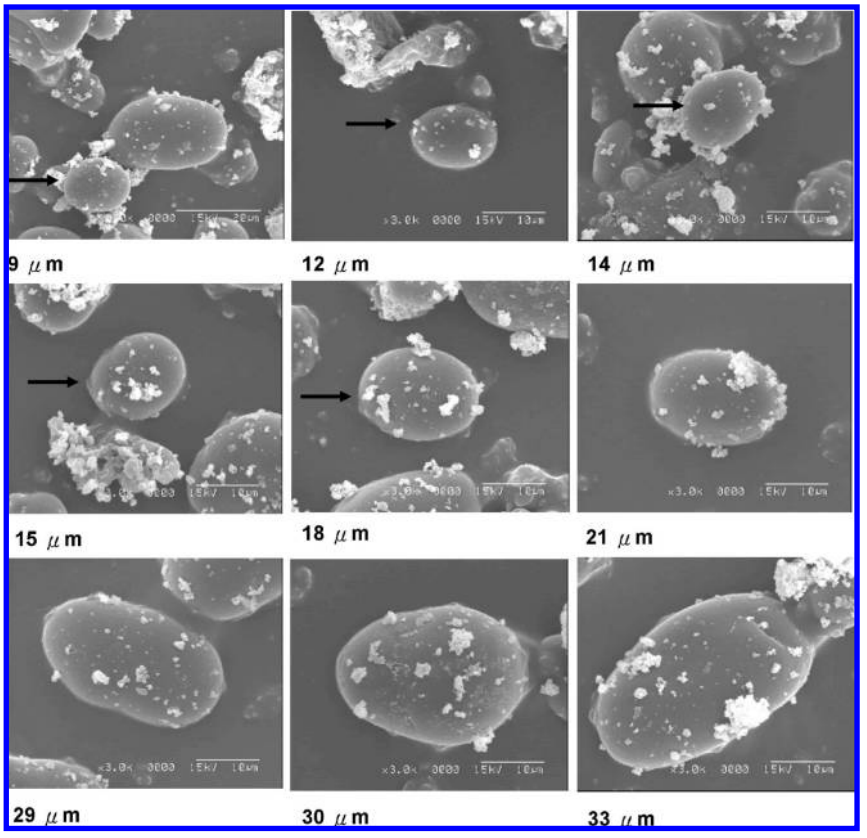

Figure 3. Scanning electron micrographs of mungbean raw starch granules of different sizes. Arrows point to the granule of the size in focus.

preparations, the SGAPs included many proteins in the crude granule preparation (**Figures 6** and **7**). When the crude and the washed granules were compared, the major 64-, 59-, 51-, 26-kDa species found in the crude granules (**Figure 6A**) were not obvious in the washed granule preparation (**Figure 6B**), indicating that these four species were peripherally and loosely bound surface SGAPs. When the raw and washed granules were further refined with TCA treatment, it showed that in addition to the above 4 major proteins, the 48-, 39-, 33-, and 32-kDa species were enriched in the raw-refined granules (**Figure 7**, the 2 left Raw-refined lanes). On the contrary, only a major 58-kDa protein and the 31-, 29-, and 27-kDa species were left in the washed-refined granules (**Figure 7**, the 2 right Washed-refined lanes). In addition, when the washed (**Figure 6B**) and washed-refined (**Figure 7**, the 2 right lanes) SGAP profiles were compared, it showed that the 98-, 84-, 57-, 43-, 35−38- kDa species were removed by TCA treatment, indicating that they were tightly bound SGAP species. Therefore, it clearly demonstrated that the washing and refining procedures acted progressively to remove surface proteins and tightly bound proteins on the starch granules, retaining only 1 major integral protein in the mungbean starch (**Figure 7**). The CsCl-washed−TCA-refined mungbean starch granules had the highest purity of any of the treatments.

Identification of the Major Integral SGAP in Mungbean Starch Granules. The identity of the major integral 58-kDa protein in mungbean starch granules was examined by in-gel trypsin digestion, followed by MALDI-TOF mass spectrometry and database search. The spectrum shown in **Figure 8** displayed the tryptic peptide mass fingerprint of the 58-kDa protein. The monoisotoped mass values of these tryptic fragments were subjected to Mascot search. All of the top 50 matched proteins were identical to granule-bound starch synthase (GBSS). Although the matched GBSS was published mostly in partial size (such as score 81, gi/115361913 *Sorbus americana* of approximately 35 kDa; and score 55, gi/83755343 *Dunalia brachyacantha* of approximately 27-kDa), two GBSS species had a molecular size similar to that of the 58-kDa protein. The GBSS Ia precursor from cowpea (*Vigna unguiculata*, gi/145202752, mass 67 kDa), had the highest matched score of 167. These peptide queries matched 19 out of 52 searched tryptic peptides and with a sequence coverage of 35%. The GBSS precursor from *Zea mays* (gi/33321047, mass 66 kDa) had the second highest score of 52. The peptide queries from *Zea mays* matched 9 out of 52 searched tryptic peptides. Nevertheless, the mature maize GBSS has been reported of a 58-kDa protein (*18*). **Table 1** summarizes the internal sequences of mungbean GBSS and the corresponding matched regions in cowpea GBSS Ia. The MALDI-TOF data thus confirmed the major 58-kDa protein, tightly associated inside mungbean starch granules, to be mungbean GBSS I.

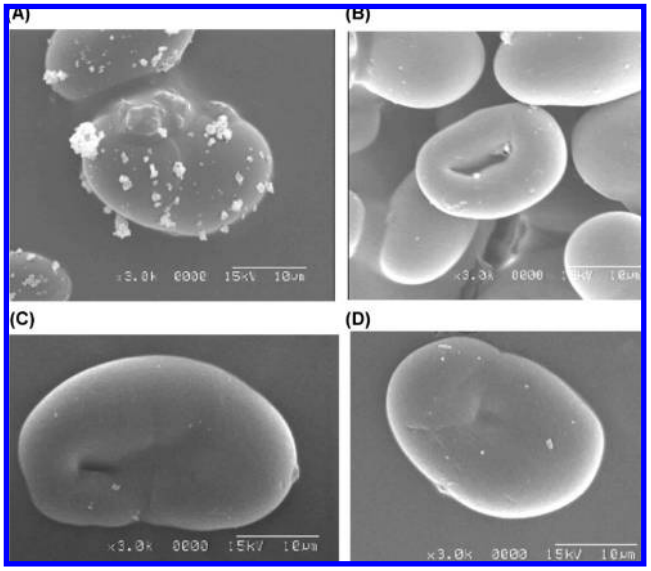

Figure 4. Scanning electron micrographs of abnormal surface features on some granules. (**A**) The tumor-like surface; (**B**) the dented hole; (**C**) the fissured slot; (**D**) the growth-line surface.

DISCUSSION

Starch granules from different botanical origin differ in morphology of shape and size. On the basis of size, they are generally classified into monomodal (potato, maize, and cassava), bimodal (wheat, barley, and rye), small granules (0.5−15 μm in oats and rice), and very small (0.3−4 μm in taro and amaranth) types. Genotypes of one source affect the proportion of these granule size populations (*19*). Mungbean KPS1 starch granule size (**Figure 3**) showed that most large granules were ovoid and that the small ones (< 15 μm) were spherical, a seemingly bimodal type distribution (*16*). In the bimodal type of wheat starch, A (large)-granules and B (small)-granules are biosynthesized at two different stages of development. A-granules are formed when the endosperm cells are dividing; B-granules are initiated during the endosperm enlargement stage (*4*). Questions naturally emerged, such as how are the morphogenesis and biogenesis of the small spherical and large ovoid granule populations in mungbean starch granules, are they generated from different sets of biosynthetic enzymes, are they produced in different amyloplast compartments of the bean cells, and are the small spherical granules the initially produced species by one set of enzymes, which grow and elongate into the ovoid shapes by another set of enzymes?

The unusual appearance on a few mungbean granules was also revealed such as the tumor-like surface, dented hole, fissured slot,

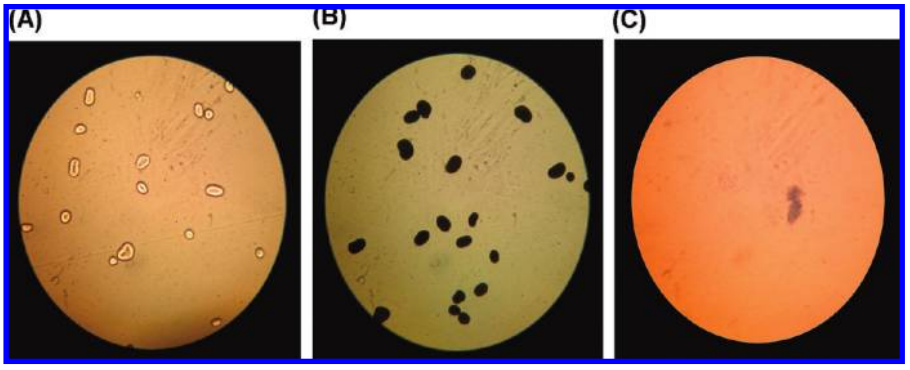

Figure 5. Comparison of the iodine stained raw starch and gelatinized starch granules under a light microscope. (**A**) Unstained raw starch granule; (**B**) stained raw starch granules; (**C**) stained gelatinized starch granules. Samples were magnified 400×.

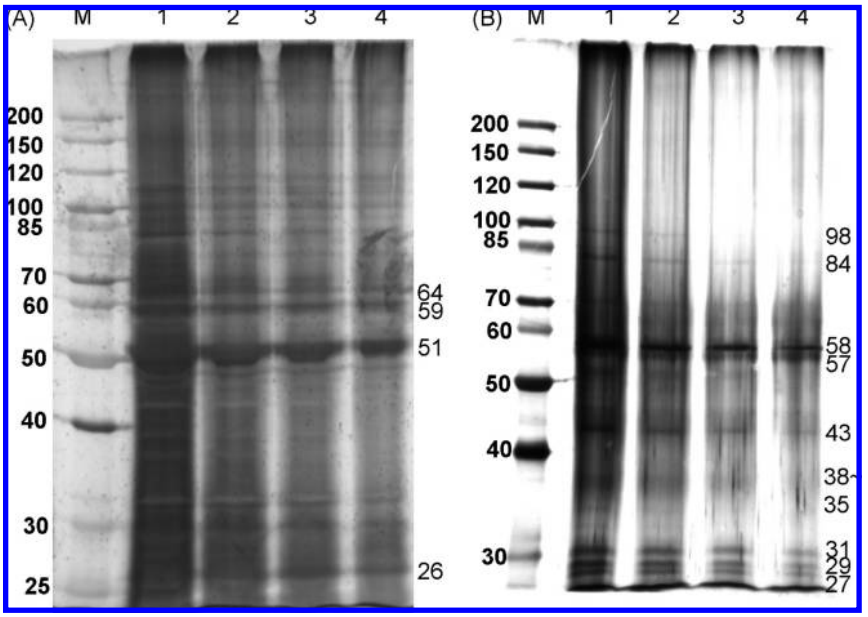

Figure 6. SGAP profiles of the raw and washed starch granules. (**A**) Proteins of the raw granule. Lanes 1−4 were loaded with 20 μL, 10 μL, 5 μL, and 2.5 μL of the extracted protein supernatant from 5 mg of granules in 100 μL of sample buffer after boiling for 2-min. (**B**) Proteins of the washed granule. Lanes 1−4 were loaded with 12 μL, 6 μL, 3 μL, and 1.5 μL of the extracted protein supernatant from 3 mg of granules in 60 μL of sample buffer after boiling for 2 min. Lane M is the molecular weight marker. The sample volume of each well was compensated with sample buffer to apply an equal amount of 20 μL.

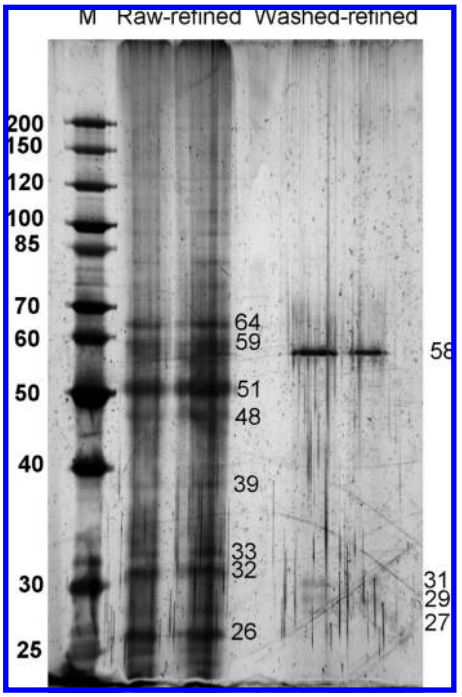

Figure 7. SGAP profiles of the raw and washed starch granules after TCA treatment. Left two lanes: proteins extracted from TCA-refined raw granule (the Raw-refined). Right two lanes, proteins extracted from TCA-refined washed granule (the Washed-refined). Protein was extracted from 3 mg of refined granules in 60 μL of sample buffer by boiling for 5 min. Then 20 μL of the supernatant was taken for each lane analysis. Lane M is the molecular weight marker.

or growth-line on the ovoid granule surface (**Figure 4**). This abnormal appearance on the large starch granules of the smooth pea has also been reported to show a cracked appearance with internal fissures (*20*). The characteristics of bean and pea starch granules have been described as thick disks with a cut around the middle or at the ends and an indentation at one end (*21*). Nevertheless, some environmental factors, biosynthesis, and the genetics aspects, were reviewed recently (*19*), and have been shown to determine the starch granule's size in higher plants. It would be interesting to study the effects of these factors on mungbean starch granule morphology in the future.

SDS−PAGE analysis (**Figures 6** and **7**) demonstrated that the crude granules were contaminated with a lot of surface proteins. The CsCl-washed granules contained only tightly bound proteins, and the washed TCA-refined granules retained only the integral granule proteins. The CsCl-washed preparation was modified from a previous method (*13*) using a two-stage treatment: after the 80% (w/v) CsCl wash to remove all less dense material, storage proteins, and surface proteins and the further removal of those trace surface proteins and lipids with 0.5% sodium laurate (*22*). The 0.5% sodium laurate was replaced by 1% SDS in this study. Therefore, after removing surface proteins, the SGAP profile of the washed granules enriched the major 58-, 57-kDa species and the minor 98-, 84-, 43-, 35−38, - 31-, 29-, and 27-kDa species (**Figure 6B**). TCA treatment on the CsCl-washed granules showed that these tightly bound granule proteins were removed. TCA is widely used as a protein precipitating/solubilizing agent. The action of TCA was possibly due to its hydrophobic trichloromethyl group that gains access to the interface between SGAP and starch granules, and its negative charge further disturbs the electrostatic attraction between the amino acid of the protein molecule and the glucosyl moiety of starch molecule that solubilized the lightly bound granule proteins. The CsCl-washing followed by TCA-treatment provided a suitable protocol for the preparation of high purity mungbean starch granules and for analyzing their SGAPs.

From the literature, the majority of SGAPs are believed to be starch biosynthetic enzymes, which are trapped during the development of the granular structure, but some SGAPs serve as supplementary storage proteins. At least 10 kinds of SGAPs with molecular size ranging from 5 to 149 kDa can be found in most starch granules. GBSS is known to be one of the most important SGAPs in terms of its quantity in the granules (*7*). GBSS is an isoform of starch synthase (SS; ADP-glucose: α-1,4-glucan 4-α-glucosyltransferase; EC 2.4.1.21) that is exclusively bound to the starch granule. It catalyzes the transfer of the glucosyl moiety

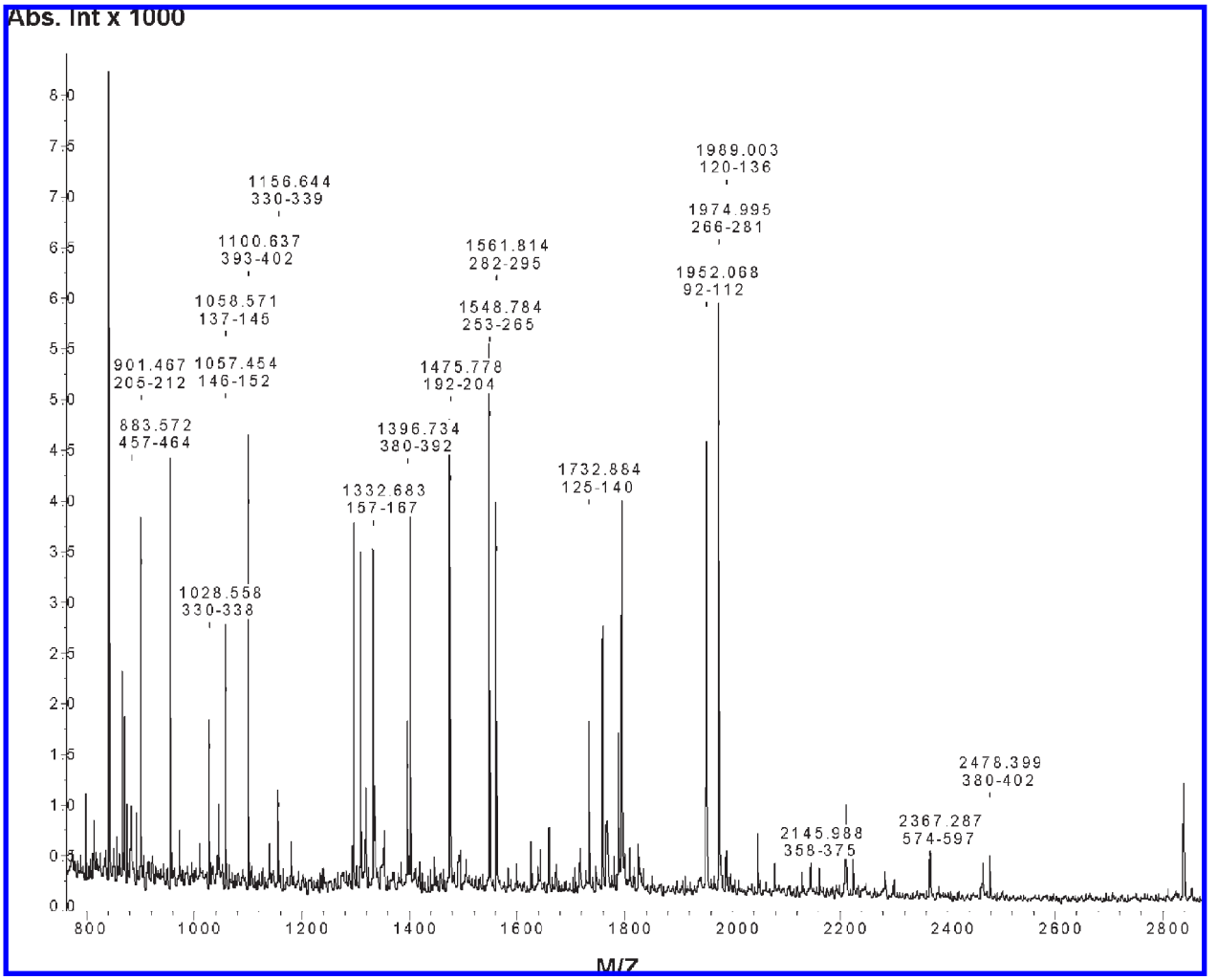

Figure 8. Tryptic peptide mass fingerprint of the major integral 58-kDa protein in mungbean starch granule derived from MALDI-TOF analysis. The upper values indicate the observed mass peaks. The lower values labeled the corresponding regions in gi 145202752 protein (**Table 1**).

Table 1. Sequence and Matched Regions of the Tryptic Fragment Mass of the 58-kDa Protein with Cowpea Granule-Bound Starch Synthase Ia Precursor (*Vigna unguiculata*, gi 145202752; Mass, 67287 Da; Mascot Search Score, 167)

start - end	observed *m*/*z*	sequence
92 - 112	1952.1	KTGGLGDVLGGLPPALAGHGHRV
120 - 136	1989.0	RYDQYKGAWDTSVTVEVKV
125 - 140	1732.9	KGAWDTSVTVEVKVADRI
137 - 145	1058.6	KVADRIETVRF
146 - 152	1057.5	RFFHCYKRG
157 - 167	1332.7	RVFVDHPCFLAKV
192 - 204	1475.8	RFSLLCQAALEAPRV
205 - 212	901.5	RVLNLNSNKY
253 - 265	1548.8	KVAFCIHNIAYQGRH
266 - 281	1975.0	RHAFEDFPLLNLPNEYRS
282 - 295	1561.8	RSAFDFTDGHLKPVKG
330 - 338	1028.6	RGVELDNIIRK
330 - 339	1156.6	RGVELDNIIRKN
358 - 375	2146.0	KTDKFIDMHFDTTTVMEAKC
380 - 392	1396.7	KEALQAEVGLPVDRD
380 - 402	2478.4	KEALQAEVGLPVDRDIPLIGFIGRL
393 - 402	1100.6	RDIPLIGFIGRL
457 - 464	883.6	KFNGPLAHKI
574 - 597	2367.3	KVLLSLDVAGSEAGIEGDEIAPLAKE

from ADP-glucose to the nonreducing end of an α-1,4-glucan. The role of GBSS involved in the synthesis of amylose was studied from amylose free (waxy) mutants that lack the 58−60 kDa GBSS protein. GBSS is often referred to as the waxy protein where GBSS mutations resulted in an opaque endosperm that was described as smooth, firm, and containing noncorneous starch (*23*). GBSS from higher plants have molecular sizes of approximately 60-kDa encoded at the *WAXY* loci of cereals, the *AMF* locus of potato, and the *LAM* locus of pea (*24*). GBSS was reported as the only starch synthase found exclusively within the granule and is responsible for the synthesis of amylose (*25*). Mungbean granules also showed that the integral 58-kDa GBSS was the most abundant protein (**Figure 7**). Previously, we have detected high starch synthase activities (13 U/g starch) of the raw starch granules and in the amylase-digested lysate of the granules (15.8 U/mL) in this mungbean KPS1 var. (*11*). The 58-kDa GBSS identified in this study should mainly correspond to these enzyme activities.

The internal amino acid sequences of mungbean GBSSI were revealed by MALDI-TOF. It showed that the tryptic fragments of the 58-kDa protein matched 19 out of the searched 52 tryptic peptide fragments with a sequence coverage of 35% to GBSS Ia precursor of cowpea (gi /145202752) (**Table 1**). Seven fragments with observed *m*/*z* values of 1057, 1475, 901, 1548, 1028, 1156, and 1396 were found in most matched GBSS proteins, indicating that they are highly conserved sequences in plant GBSS I. Because of the fact that the GBSS I gene is a single copy in cereals and dicots, the flanking regions of these conserved sequences have been useful in designing GBSS-gene-specific primers to amplify GBSS gene fragments and use their sequences to perform cladistic analysis and establish evolutionary relationships among plant

species (*26*). Additionally, within the *m*/*z* 1952 tryptic fragment located at the N-terminal of the 58-kDa, the identified residues KTGGL constitute a conserved motif that was also found in most known GBSS I and soluble starch synthase varieties. KTGGL is thought to be the binding site for the substrate ADP-glucose (*27*). Therefore, these internal amino acid sequences provided valuable information for us to further investigate mungbean GBSS I at the molecular level.

In conclusion, using the popular mungbean cultivar KPS1, this study enhanced our understanding of the morphology and SGAP of mungbean starch granules. Granules observed under SEM showed bimodal populations of spherical and ovoid shapes. Starch granules with high purity were obtained by a CsCl-washing plus TCA-treatment protocol. In conjunction with SGAP analysis, surface protein, tightly bound protein, and integral protein species were revealed. The major 58-kDa integral protein was identified as mungbean granule-bound starch synthase I.

LITERATURE CITED

(1) Yan, T. L.; Li, A. Z.; Zhao, B. Y. Correlation between seed gloss and qualitative characters in mung bean. *Acta Agric. Boreali-Sin.* **1991**, *6*, 96–98.

(2) Hoover, R.; Li, Y. X.; Hynes, G.; Senanayake, N. Physicochemical characterization of mung bean starch. *Food Hydrocoll.* **1997**, *11*, 401–408.

(3) Oates, C. G. Fine-structure of mung bean starch - an improved method of fractionation. *Starch* **1990**, *42*, 464–467.

(4) Buleon, A.; Colonna, P.; Planchot, V.; Ball, S. Starch granules: structure and biosynthesis. *Int. J. Biol. Macromol.* **1998**, *23*, 85–112.

(5) Davis, J. P.; Supatcharee, N.; Khandelwal, R. L.; Chibbar, R. N. Synthesis of novel starches in Planta: opportunities and challenges. *Starch* **2003**, *55*, 107–120.

(6) Lii, C.-Y.; Chu, Y.-L.; Chang, Y.-H. Isolation and characterization of mungbean starch. *Asian Vegetable Research and Development Center, Bangkok,Thailand* **1987**, 528–535.

(7) Baldwin, P. M. Starch granule-associated proteins and polypeptides: A review. *Starch* **2001**, *53*, 475–503.

(8) Han, X. Z.; Campanella, O. H.; Guan, H. P.; Keeling, P. L.; Hamaker, B. R. Influence of maize starch granule-associated protein on the rheological properties of starch pastes. Part I. Large deformation measurements of paste properties. *Carbohydr. Polym.* **2002**, *49*, 315–321.

(9) Shotwell, M. A.; Larkins, B. A. The Biochemistry and Molecular Biology of Seed Storage Proteins. In *The Biochemistry of Plants*; Marcus, A., Ed.; Academic Press: San Diego, CA, 1989; Vol. *15*, pp 297−345.

(10) Chiang, W. C. A new process for separation of mungbean starch. *Food Sci.* **1983**, *10*, 1–10.

(11) Ko, Y.-T.; Chang, S.-K.; Chen, H.-C.; Li, Y.-C. GBSS activities on mungbean (*Vigna radiata L.*) starch granule and analysis of its total protein profiles. *Taiwan J. Agric. Chem. Food Sci.* **2004**, *42*, 132–139.

(12) Edwards, A.; Marshall, J.; Sidebottom, C.; Visser, R. G. F.; Smith, A. M.; Martin, C. Biochemical and molecular characterization of a novel starch synthase from potato-tubers. *Plant J.* **1995**, *8*, 283–294.

(13) South, J. B.; Morrison, W. R. Isolation and analysis of starch from single kernels of wheat and barley. *J. Cereal Sci.* **1990**, *12*, 43–51.

(14) Srinives, P.; Sorajjapinun, W.; Koizumi, M. In *Mungbean Breeding Research at ARC-AVRDC/KU*, The 4th National Mungbean Workshop, Nanjing City, Jiangsu Province, China, Sept 7−10, 2001.

(15) Ko, Y. T.; Pan, C. H.; Lee, Y. T.; Chang, J. Y. Detection of proteins related to starch synthase activity in the developing mungbean (*Vigna radiata L.*). *J. Agric. Food Chem.* **2005**, *53*, 4805–4812.

(16) Chang, Y. H.; Lin, C. L.; Chen, J. C. Characteristics of mung bean starch isolated by using lactic acid fermentation solution as the steeping liquor. *Food Chem.* **2006**, *99*, 794–802.

(17) Yajima, H.; Nishimura, T.; Ishii, T.; Handa, T. Effect of concentration of iodide on the bound species of I_2/I_3^- in the amylose iodine complex. *Carbohydr. Res.* **1987**, *163*, 155–167.

(18) Shure, M.; Wessler, S.; Fedoroff, N. Molecular-identification and isolation of the waxy locus in maize. *Cell* **1983**, *35*, 225–233.

(19) Lindeboom, N.; Chang, P. R.; Tyler, R. T. Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches: A review. *Starch* **2004**, *56*, 89–99.

(20) Bertoft, E.; Manelius, R.; Zhu, Q. Studies on the structure of pea starches 0.1. Initial-stages in alpha-amylolysis of granular smooth pea starch. *Starch* **1993**, *45*, 215–220.

(21) Jane, J. L.; Kasemsuwan, T.; Leas, S.; Zobel, H.; Robyt, J. F. Anthology of starch granule morphology by scanning electron-microscopy. *Starch* **1994**, *46*, 121–129.

(22) Sulaiman, B. D.; Morrison, W. R. Proteins Associated with the surface of wheat-starch granules purified by centrifuging through cesium-chloride. *J. Cereal Sci.* **1990**, *12*, 53–61.

(23) Kossmann, J.; Lloyd, J. Understanding and influencing starch biochemistry. *Cri. Rev. Biochem. Mol. Biol.* **2000**, *35*, 141–196.

(24) Smith, A. M.; Denyer, K.; Martin, C. The synthesis of the starch granule. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1997**, *48*, 65–87.

(25) Jobling, S. Improving starch for food and industrial applications. *Cur. Opin. Plant Biol.* **2004**, *7*, 210–218.

(26) Campbell, C. S.; Evans, R. C.; Morgan, D. R.; Dickinson, T. A.; Arsenault, M. P. Phylogeny of subtribe Pyrinae (formerly the Maloideae, Rosaceae): Limited resolution of a complex evolutionary history. *Plant System. Evol.* **2007**, *266*, 119–145.

(27) Nakamura, T.; Vrinten, P.; Hayakawa, K.; Ikeda, J. Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. *Plant Physiol.* **1998**, *118*, 451–459.

Received for Review January 13, 2009. Revised manuscript received March 8, 2009. Accepted March 31, 2009. This work was supported by NSC95-2313-B-019-023-MY2 and partially by a summer undergraduate project funded by the Plan to Encourage Teaching Excellence in Universities.